AMENDMENTS TO THE SPECIFICATION

Please amend the paragraphs relating to Figures 2-4, which span pages 10-14 and begin under the Brief Description of the Drawings section on page 10, as follows:

~~Figure~~ Figures 2A-D show results of ~~relates to~~ a sensitive assay for Shh signaling in NIH-3T3 cells. ~~(A) Purification of cholesterol- and palmitate-modified mouse Sonic hedgehog signaling domain $ShhN_p$. Detergent-insoluble proteolipid complexes were isolated from 293 cells expressing full-length Shh (M.-K. Cooper, J. A. Porter, K. E. Young, P. A. Beachy, *Science* **280**, 1603 (1998)), and $ShhN_p$ was purified to apparent homogeneity by immunoaffinity chromatography. Although recombinant ShhN lacking cholesterol and palmitate modifications is fully active in neural plate explant culture assays, this form of ShhN was poorly active in the NIH-3T3 cells. We therefore used the detergent insolubility of cholesterol-modified ShhN and affinity chromatography to purify the processed ShhN protein ($ShhN_p$) from a human 293 cell line engineered to express the full-length mouse Shh construct. Detergent-insoluble complexes (DIGs) were isolated as described by Brown and Rose (1992), with the following modifications. Cells from a 150 mm dish were lysed and collected in 2 mL of lysis solution (10 mM $NaHPO_4$, pH 6.5, 150 mM NaCl, 0.5 mM PMSF, 1% Triton X-100, 2 μg/ml Pepstatin A, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 2 μg/ml E64) at 4 °C. Eight sucrose density steps (35.625-5%, 4.375%/step; made in above solution, without detergent) were layered onto the 40% sucrose lysate and centrifugation proceeded for 2-12 hr. Low-density, flocculent material was collected (from original position of 18.125% step), diluted 5-fold in 10 mM $NaHPO_4$, pH 6.5, 150 mM NaCl and harvested by centrifugation at 20,000 x g for 15 min (all at 4 °C). Complexes were solubilized at ambient temperature in 1% n-octyl-α-D-glucopyranoside, 50 mM HEPES, pH 7.5, 150 mM NaCl and the single $ShhN_p$ immunoaffinity purification step proceeded essentially as described in Pepinsky et al. (1998), except the anti-Shh-N monoclonal antibody 5E1 was coupled to Affi-Gel 10 (Bio-Rad) at 6.6 mg/ml gel. The mass of the purified species as determined by mass spectrometry corresponds to that of the ShhN polypeptide bearing covalent palmitoyl and cholesteryl adducts: murine ShhN polypeptide, 19,574.05 Da; esterified cholesterol, 368.65; palmitoyl mass (in ester or amide linkage), 238.42; sum, 20,181.1. The inset shows samples from lysate, detergent-insoluble glycolipid complexes (DIGs; 8 lysate sample equivalents), and~~

purified $ShhN_p$ (0.75 μg) as separated in SDS-PAGE (14%) and stained with Coomassie blue. Mass standards migrated as indicated. (**B**) NIH-3T3 cells respond to $ShhN_p$. NIH-3T3 cells cotransfected with Gli-luc reporter and TK-promoter-driven Renilla luciferase control were treated with the indicated concentrations of $ShhN_p$ for 2 days. Confluent cultures of NIH-3T3 cells were plated at 1:6 dilution to 24 or 96-well plates. On the following day, the cells were transfected with renilla luciferase (pRL-TK or pRL-SV40; Clontech) or β-galactosidase transfection control (10% w/w DNA), Gli-Luc reporter (40%) and the constructs indicated (50%) using Fugene 6 (Roche) transfection reagent (250 ng (24 well plate) or 100 ng (96-well plate) DNA/well, 3:1 ratio (v/w) of reagent to DNA). After the cells had reached saturation density (1-2 d), they were changed to low serum medium (0.5% bovine calf serum), and treated with the reagents indicated for 1-2 d. Firefly and Renilla luciferase and β-galactosidase activities were assayed from the cell lysates by luminometry using dual-luciferase (Promega) and Galacto-Light (Tropix) kits, respectively. Luciferase activities are normalized relative to control; a representative experiment is shown. Note that in this and all subsequent reporter assays, TK-Renilla luciferase activity is used as a control for normalization. (**C**) Shh pathway activation is sensitive to cyclopamine in NIH-3T3 cells. NIH-3T3 cells transfected as above (in triplicate) were treated with $ShhN_p$ (4 nM) and/or cyclopamine (5 μM) for 2 d as indicated. Normalized luciferase activities are given as fold induction relative to control. Error bars indicate one standard deviation. (**D**) Low cell density inhibits Shh pathway activity downstream of Smo. Cultures of Shh-LIGHT (open boxes) or SmoA1-LIGHT (filled diamonds) cells were plated to 96-well plates in a series of twofold dilutions. The NIH-3T3 cell clone Shh-LIGHT and Shh-LIGHT2 stably incorporating the Gli-luc reporter and TK-renilla vectors were established by cotransfection with a vector encoding G418 resistance (pSV-Neo), followed by selection with G418 and cell cloning. Subsequently, a clonal subline of Shh-LIGHT expressing activated Smo (SmoA1-LIGHT) was established using an expression vector that allows hygromycin selection (pcDNA3.1+ hygro; Invitrogen). The expression of SmoA1 in the cell line was verified by immunoblotting. The Shh-LIGHT cells were treated with 4 nM $ShhN_p$, and Gli-luciferase reporter activity was assayed after 24 h. Fold induction of the reporter (% of maximum is relative to equally dense Shh-LIGHT control culture) and cell densities (% of maximum Renilla luciferase activity) were measured at the end of the experiment. Error bars indicate one standard deviation (quadruplicate wells).

~~Figure~~ Figures 3A-C ~~demonstrate~~ demonstrates how cyclopamine acts by inhibiting the activity of Smo. ~~(A) *Ptc1 -/-* cells are sensitive to cyclopamine. Fibroblasts from *Ptc1 -/-* embryos were treated with cyclopamine or forskolin as indicated. After 3 d, cells were lysed and β-galactosidase activity relative to protein concentration was measured. Since β-galactosidase is expressed from the *Ptc1* locus, its expression reflects the activity of the Shh pathway. A representative experiment is shown. (B) Activated mutants of Smo are resistant to cyclopamine. Cultures of NIH-3T3 cells were transfected (in triplicate) with Gli-luciferase reporter, TK-Renilla luciferase control vector and Smo or SmoA1 expression vectors. Smo DNA was used at 50% w/w, and SmoA1 at 50%, 5%, and 0.5% w/w. Subsequently, the cultures were treated with 5 μM cyclopamine for 2 d. Error bars indicate one standard deviation. The leftmost four bars, shown for comparison, are as in Fig. 2C. (C) High level expression of Ptc1 restores cyclopamine resistant response of SmoA1 to $ShhN_p$. NIH-3T3 cells were transfected with Gli-luc reporter, TK-Renilla, Ptc1CTD and SmoA1 expression vectors (Ptc to Smo DNA ratio = 9). Subsequently, the cultures were treated with $ShhN_p$ (2 nM), cyclopamine (5 μM) and/or forskolin (100 μM) as indicated for 2 d. Note that SmoA1 activation of pathway is dramatically reduced by high levels of Ptc1 activity (compare to panel B), and that 2 nM $ShhN_p$ restores pathway activity even in the presence of 5 μM cyclopamine. A representative experiment is shown. (D) Tumor-derived mutant Smo proteins are intrinsically more active than wild type Smo. NIH-3T3 cells were cotransfected with Gli-luc reporter, β-galactosidase transfection control, and a control vector or an expression vector encoding the indicated Smo-Renilla luciferase fusion protein. In the representative experiment shown, Shh pathway activity and Smo protein levels were measured as firefly and Renilla luciferase activities relative to β-galactosidase, respectively. Epitope-tagged Smo and activated Smo proteins also displayed similar levels of expression in these cells (not shown).~~

~~Figure~~ Figures 4A-B ~~depict~~ depicts a cyclopamine derivative of increased potency. ~~(A) 3-Keto, N-aminoethyl aminocaproyl dihydrocinnamoyl cyclopamine (KAAD cyclopamine) was synthesized from cyclopamine. Structure of KAAD cyclopamine was verified by NMR and mass spectrometry analyses. KAAD cyclopamine can block pathway activation by tumor-derived Smo. Shh-LIGHT2 (diamonds) and SmoA1-LIGHT (circles) cells were treated with 4 nM $ShhN_p$ (Shh-LIGHT2) and increasing concentrations of KAAD cyclopamine (both lines) for 2 d. Relative reporter activity is normalized to maximum. Note the increased inhibitory potency of~~

~~KAAD-cyclopamine as compared to cyclopamine in Fig. 3 A-C. (B) KAAD-cyclopamine can block pathway activation in *Ptc1 -/-* cells. p2$^{PTC -/-}$ cells (these cells are a cloned line derived from *Ptc -/-* mouse embryonic fibroblasts) were treated with increasing concentrations of cyclopamine (open boxes) or KAAD-cyclopamine (filled boxes) for 2d. The suppression of pathway activity induced by SmoA1-Renilla by high concentrations of cyclopamine derivatives did not involve a decrease in the level of expression of the Smo construct (not shown). Cells were seeded into duplicate 96-well plates, allowed to grow to saturation density, and incubated with cyclopamine and KAAD-cyclopamine for 2 d. β-galactosidase activity was determined using Galacto-Light kit (no inactivation of endogenous β-gal activity, Tropix). β-galactosidase activities were normalized to cell mass as determined from a treated duplicate plate using the Cell Titer 96AQ assay (Promega). Maximum normalized β-galactosidase activities (1103 for KAAD-cyclopamine and 916 for cyclopamine) were set to 1 and minimum activities (191 and 144, respectively) were set to 0. Significant toxicity (microscopically visible cell death, or decrease in Cell Titer reading) was not observed, even at the highest doses of compounds used. β-galactosidase activity is normalized to the maximum. Error bars in **A** and **B** indicate one standard deviation.~~

On page 91, please insert the following paragraph between the paragraph ending with "acts" on line 2 and the paragraph beginning with "Cellular" on line 3:

Figures 2A-D show results of a sensitive assay for Shh signaling in NIH-3T3 cells. (**A**) Purification of cholesterol- and palmitate-modified mouse Sonic hedgehog signaling domain $ShhN_p$. Detergent-insoluble proteolipid complexes were isolated from 293 cells expressing full-length Shh (M. K. Cooper, J. A. Porter, K. E. Young, P. A. Beachy, *Science* **280**, 1603 (1998)), and $ShhN_p$ was purified to apparent homogeneity by immunoaffinity chromatography. Although recombinant ShhN lacking cholesterol and palmitate modifications is fully active in neural plate explant culture assays, this form of ShhN was poorly active in the NIH-3T3 cells. We therefore used the detergent insolubility of cholesterol-modified ShhN and affinity chromatography to purify the processed ShhN protein ($ShhN_p$) from a human 293 cell line engineered to express the full length mouse Shh construct. Detergent-insoluble complexes (DIGs) were isolated as

described by Brown and Rose (1992), with the following modifications. Cells from a 150 mm dish were lysed and collected in 2 mL of lysis solution (10 mM $NaHPO_4$, pH 6.5, 150 mM NaCl, 0.5 mM PMSF, 1% Triton X-100, 2 μg/ml Pepstatin A, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 2 μg/ml E64) at 4 °C. Eight sucrose density steps (35.625-5%, 4.375%/step; made in above solution, without detergent) were layered onto the 40% sucrose lysate and centrifugation proceeded for 2-12 hr. Low-density, flocculent material was collected (from original position of 18.125% step), diluted 5-fold in 10 mM $NaHPO_4$, pH 6.5, 150 mM NaCl and harvested by centrifugation at 20,000 x g for 15 min (all at 4 °C). Complexes were solubilized at ambient temperature in 1% n-octyl-α-D-glucopyranoside, 50 mM HEPES, pH 7.5, 150 mM NaCl and the single $ShhN_p$ immunoaffinity purification step proceeded essentially as described in Pepinsky et al. (1998), except the anti-Shh-N monoclonal antibody 5E1 was coupled to Affi-Gel 10 (Bio-Rad) at 6.6 mg/ml gel. The mass of the purified species as determined by mass spectrometry corresponds to that of the ShhN polypeptide bearing covalent palmitoyl and cholesteryl adducts: murine ShhN polypeptide, 19,574.05 Da; esterified cholesterol, 368.65; palmitoyl mass (in ester or amide linkage), 238.42; sum, 20,181.1. The inset shows samples from lysate, detergent-insoluble glycolipid complexes (DIGs; 8 lysate sample equivalents), and purified $ShhN_p$ (0.75 μg) as separated in SDS-PAGE (14%) and stained with Coomassie blue. Mass standards migrated as indicated. (**B**) NIH-3T3 cells respond to $ShhN_p$. NIH-3T3 cells cotransfected with Gli-luc reporter and TK promoter-driven Renilla luciferase control were treated with the indicated concentrations of $ShhN_p$ for 2 days. Confluent cultures of NIH-3T3 cells were plated at 1:6 dilution to 24 or 96-well plates. On the following day, the cells were transfected with renilla luciferase (pRL-TK or pRL-SV40; Clontech) or β-galactosidase transfection control (10% w/w DNA), Gli-Luc reporter (40%) and the constructs indicated (50%) using Fugene 6 (Roche) transfection reagent (250 ng (24 well plate) or 100 ng (96 well plate) DNA/well, 3:1 ratio (v/w) of reagent to DNA). After the cells had reached saturation density (1-2 d), they were changed to low serum medium (0.5% bovine calf serum), and treated with the reagents indicated for 1-2 d. Firefly and Renilla luciferase and β-galactosidase activities were assayed from the cell lysates by luminometry using dual luciferase (Promega) and Galacto-Light (Tropix) kits, respectively. Luciferase activities are normalized relative to control; a representative experiment is shown. Note that in this and all subsequent reporter assays, TK-Renilla luciferase activity is used as a

control for normalization. (**C**) Shh pathway activation is sensitive to cyclopamine in NIH-3T3 cells. NIH-3T3 cells transfected as above (in triplicate) were treated with $ShhN_p$ (4 nM) and/or cyclopamine (5 μM) for 2 d as indicated. Normalized luciferase activities are given as fold induction relative to control. Error bars indicate one standard deviation. (**D**) Low cell density inhibits Shh pathway activity downstream of Smo. Cultures of Shh-LIGHT (open boxes) or SmoA1-LIGHT (filled diamonds) cells were plated to 96-well plates in a series of twofold dilutions. The NIH-3T3 cell clone Shh-LIGHT and Shh-LIGHT2 stably incorporating the Gli-luc reporter and TK-renilla vectors were established by cotransfection with a vector encoding G418 resistance (pSV-Neo), followed by selection with G418 and cell cloning. Subsequently, a clonal subline of Shh-LIGHT expressing activated Smo (SmoA1-LIGHT) was established using an expression vector that allows hygromycin selection (pcDNA3.1+ hygro; Invitrogen). The expression of SmoA1 in the cell line was verified by immunoblotting. The Shh-LIGHT cells were treated with 4 nM $ShhN_p$, and Gli-luciferase reporter activity was assayed after 24 h. Fold induction of the reporter (% of maximum is relative to equally dense Shh-LIGHT control culture) and cell densities (% of maximum Renilla luciferase activity) were measured at the end of the experiment. Error bars indicate one standard deviation (quadruplicate wells).

On page 93, please insert the following paragraph between the paragraph ending with "(not shown)" on line 2 and the paragraph beginning with "The" on line 3:

Figures 3A-C demonstrate how cyclopamine acts by inhibiting the activity of Smo. (**A**) *Ptc1-/-* cells are sensitive to cyclopamine. Fibroblasts from *Ptc1-/-* embryos were treated with cyclopamine or forskolin as indicated. After 3 d, cells were lysed and β-galactosidase activity relative to protein concentration was measured. Since β-galactosidase is expressed from the *Ptc1* locus, its expression reflects the activity of the Shh pathway. A representative experiment is shown. (**B**) Activated mutants of Smo are resistant to cyclopamine. Cultures of NIH-3T3 cells were transfected (in triplicate) with Gli-luciferase reporter, TK-Renilla luciferase control vector and Smo or SmoA1 expression vectors. Smo DNA was used at 50% w/w, and SmoA1 at 50%, 5%, and 0.5% w/w. Subsequently, the cultures were treated with 5 μM cyclopamine for 2 d. Error bars indicate one standard deviation. The leftmost four bars, shown for comparison, are as in Fig. 2C. (**C**) High level expression of Ptc1 restores cyclopamine resistant response of SmoA1

to $ShhN_p$. NIH-3T3 cells were transfected with Gli-luc reporter, TK-Renilla, Ptc1CTD and SmoA1 expression vectors (Ptc to Smo DNA ratio = 9). Subsequently, the cultures were treated with $ShhN_p$ (2 nM), cyclopamine (5 μM) and/or forskolin (100 μM) as indicated for 2 d. Note that SmoA1 activation of pathway is dramatically reduced by high levels of Ptc1 activity (compare to panel **B**), and that 2 nM $ShhN_p$ restores pathway activity even in the presence of 5 μM cyclopamine. A representative experiment is shown.

Please amend the paragraph beginning on page 93, line 24 as follows:

To further investigate the site of cyclopamine action we transfected NIH-3T3 cells with Smo cDNA (a mouse *Smo* cDNA probe was generated using RT-PCR with degenerate oligonucleotide primers based on rat and human *Smo* sequences; this probe was subsequently used to isolate a cDNA clone containing the complete coding sequence of mouse *Smo*), and found that overexpression of Smo in the absence of Shh induces reporter expression ~10-fold. As this activation of the pathway occurs in the absence of Shh and can be suppressed by 5 μM cyclopamine (Fig. 3B), we infer that the mechanism of cyclopamine action is not direct interference with Shh binding (i.e., as a neutral antagonist of Shh). Interestingly, cyclopamine at this concentration showed little effect on reporter expression induced by the tumor-derived activated Smo mutants (Fig. 21B), suggesting the possibility that cyclopamine acts directly or indirectly upon Smo and that activating mutations render Smo proteins resistant. Cyclopamine resistance of SmoA1 also was observed at sub-maximal levels of pathway activation associated with reduced SmoA1 expression (Fig. 3B). In addition, we tested whether Shh signaling through activated Smo is affected by cyclopamine. Although activated Smo proteins previously have been reported to resist suppression by Ptc1 (M. Murone, A. Rosenthal, F. J. de Sauvage, *Current Biol.* **9**, 76 (1999)) we found that this resistance is partial, as transfection of a 9 to 1 ratio of a Ptc1 construct (not shown) or of Ptc1-CTD, a C-terminally deleted construct (Ptc1-CTD previously was shown to be expresed at higher levels than Ptc1; N. Fuse et al., *Proc Natl Acad Sci U S A* **96**, 10992 (1999)), can completely inhibit the activating effects of SmoA1 or SmoA2 (Fig. 3C). In cells thus transfected, the Gli-responsive reporter can be induced upon treatment with $ShhN_p$; induction under these circumstances is resistant to 5 μM cyclopamine (Fig. 3C), which normally would abolish Shh signaling. These results indicate that activated Smo

molecules in the presence of sufficiently high levels of Ptc1 can contribute to an essentially normal, albeit cyclopamine resistant, response to the Shh signal. The requirement for higher levels of Ptc1 is not due simply to a higher level of the Smo protein variant, as we found that the levels of wild type and activated Smo proteins produced in transfected cells were similar, despite dramatically elevated levels of reporter activity associated with activated Smo ~~(Fig. 3D)~~.

On page 94, please insert the following paragraph before the paragraph beginning with "Although" on line 23:

Figures 4A-B depict a cyclopamine derivative of increased potency. (**A**) 3-Keto, N-aminoethyl aminocaproyl dihydrocinnamoyl cyclopamine (KAAD cyclopamine) was synthesized from cyclopamine. Structure of KAAD cyclopamine was verified by NMR and mass spectrometry analyses. KAAD cyclopamine can block pathway activation by tumor-derived Smo. Shh-LIGHT2 (diamonds) and SmoA1-LIGHT (circles) cells were treated with 4 nM $ShhN_p$ (Shh-LIGHT2) and increasing concentrations of KAAD cyclopamine (both lines) for 2 d. Relative reporter activity is normalized to maximum. Note the increased inhibitory potency of KAAD cyclopamine as compared to cyclopamine in Fig. 3 A-C. (**B**) KAAD cyclopamine can block pathway activation in *Ptc1-/-* cells. $p2^{PTC-/-}$ cells (these cells are a cloned line derived from *Ptc-/-* mouse embryonic fibroblasts) were treated with increasing concentrations of cyclopamine (open boxes) or KAAD cyclopamine (filled boxes) for 2d. The suppression of pathway activity induced by SmoA1-Renilla by high concentrations of cyclopamine derivatives did not involve a decrease in the level of expression of the Smo construct (not shown). Cells were seeded into duplicate 96-well plates, allowed to grow to saturation density, and incubated with cyclopamine and KAAD cyclopamine for 2 d. β-galactosidase activity was determined using Galacto-Light kit (no inactivation of endogenous β-gal activity, Tropix). β-galactosidase activities were normalized to cell mass as determined from a treated duplicate plate using the Cell Titer 96AQ assay (Promega). Maximum normalized β-galactosidase activities (1103 for KAAD-cyclopamine and 916 for cyclopamine) were set to 1 and minimum activities (191 and 144, respectively) were set to 0. Significant toxicity (microscopically visible cell death, or decrease in Cell Titer reading) was not observed, even at the highest doses of compounds used.

β-galactosidase activity is normalized to the maximum. Error bars in **A** and **B** indicate one standard deviation.

Please amend the paragraph beginning on page 95, line 6, as follows:

The simplest explanation of cyclopamine resistance as conferred by activated Smo proteins is that cyclopamine affects Smo activity and that activating mutations render Smo proteins resistant. An alternative interpretation would be that activated Smo proteins produce a high abundance of a downstream component and that a high cyclopamine level is required to suppress the increased concentration of this downstream component. This alternative model, however, can not account for the sustained cyclopamine resistance of activated Smo proteins observed at intermediate or low levels of pathway activation (Fig. 3B) (production by activated Smo of high levels of a downstream component that is the cyclopamine target can not explain the sustained cyclopamine resistance observed at intermediate levels of pathway activation (Fig. 3B), as the hypothetical cyclopamine target in this circumstance would be present at the same moderate levels as those produced by $ShhN_p$ signaling via unaltered Smo. We also find that high levels of Smo in maximally-stimulated cells do not confer cyclopamine resistance (not shown), again inconsistent with the notion that extensive production or activation by Smo of a downstream component can confer cyclopamine resistance). As activated Smo is not expressed at higher levels than unaltered Smo ~~(Fig. 3D)~~, it would appear that activating mutations may confer a higher intrinsic ability to activate the pathway. This suggests that, like other seven transmembrane receptors (R. A. Bond et al., *Nature* **374**, 272 (1995); H. R. Bourne, *Curr Opin Cell Biol* **9**, 134 (1997)), Smo may exist in a balance between active and inactive forms. Cyclopamine and Ptc activities might shift this balance toward the inactive state and tumor-associated mutations toward the active state, thus accounting for the higher levels of Ptc and cyclopamine activity required to suppress activated Smo proteins.